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# Cryptic lineages in the Wolf Cardinalfish living in sympatry on remote coral atolls

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ABSTRACT

Coral reef health and biodiversity is under threat worldwide due to rapid climate change. However, much of the inter- and intra-specific diversity of coral reefs are undescribed even in well studied taxa such as fish. Delimiting previously unrecognised diversity is important for understanding the processes that generate and sustain biodiversity in coral reef ecosystems and informing strategies for their conservation and management. Many taxa that inhabit geographically isolated coral reefs rely on self-recruitment for population persistence, providing the opportunity for the evolution of unique genetic lineages through divergent selection and reproductive isolation. Many such lineages in corals and fish are morphologically similar or indistinguishable. Here, we report the discovery and characterisation of cryptic lineages of the Wolf Cardinalfish, *Cheilodipterus artus*, from the coral atolls of northwest Australia using multiple molecular markers from mitochondrial (CO1 and D-loop) and nuclear (microsatellites) DNA. Concordant results from all markers identified two highly divergent lineages that are morphologically cryptic and reproductively isolated. These lineages co-occurred at daytime resting sites, but the relative abundance of each lineage was strongly correlated with wave exposure. It appears, therefore, that fish from each lineage are better adapted to different microhabitats. Such cryptic and ecologically based diversity appears to be common in these atolls and may well aid resilience of these systems. Our results also highlight that underwater surveys based on visual identification clearly underestimate biodiversity, and that a taxonomic revision of the *Cheilodipterus* genus is necessary.

## 1. Introduction

Sustainable management of living systems requires an understanding of the processes that generate and sustain biodiversity in a rapidly changing climate (Bowen, 1999; Eizaguirre and Baltazar-Soares, 2014; Frankel, 1974). Phylogenetic studies of the distribution of genetic lineages among taxa (molecular systematics) and across geographic locations within taxa (phylogeography) identify appropriate evolutionarily significant units of biodiversity. Such studies also provide the evolutionary context for focusing questions on the ecological processes which generate and sustain biodiversity that are relevant to conservation management (Moritz, 2002). Coral reefs are the most diverse ecosystems in the ocean (Reaka-Kudla, 1997), but are under serious threat from a range of disturbances, especially marine heatwaves (Carpenter et al., 2008; Hoegh-Guldberg et al., 2007; Hoey et al., 2016; Hughes et al., 2017). Much of coral reef diversity is still undescribed even in the well-studied taxa of hard corals and fishes (e.g. Schmidt-Roach et al., 2014; Von Der Heyden, 2011). Therefore, in the face of imminent loss, describing and delimiting previously unrecognised diversity is imperative for informing practical management strategies to sustain the health of coral reef ecosystems (Rocha et al., 2007; van Oppen and Gates, 2006; von der Heyden, 2017).

Coral reef fishes form a conspicuous, highly speciose and important functional component of the biodiversity of coral reef ecosystems (Rocha and Bowen, 2008). Consequently, fishes are the best-studied taxa on coral reefs (Fisher et al., 2011), and it is generally accepted that most large-bodied fish have been described. However, cryptic (or sibling) species that lack clear discriminating morphological characters are generally common in marine taxa (Knowlton, 1993), including in marine (Zemlak et al., 2009) and especially coral reef fish (e.g. DiBattista et al., 2017; Fernandez-Silva et al., 2015; Priest et al., 2016; Victor, 2015). Thus, the identification of species with conservative

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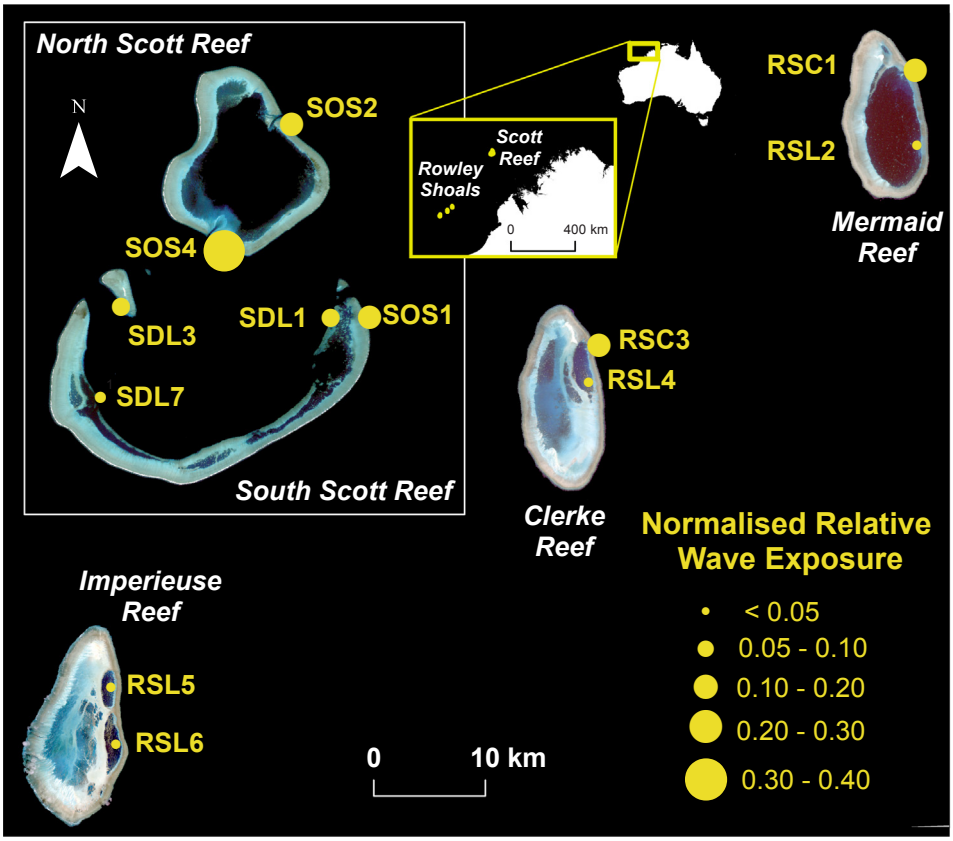


**Fig. 1.** Location of sampling sites of *Cheilodipterus artus* from Scott Reef and Rowley Shoals in northwest Australia. Relative wave exposure at each site is indicated by the size of the yellow circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

morphologies is one of the most important, although sometimes unexpected, contributions of phylogenetic studies to the conservation of coral reefs (Rocha et al., 2007).

Many taxa that inhabit coral reefs rely on self-recruitment to sustain their populations (Andutta et al., 2012; Figueiredo et al., 2013; McCook et al., 2009). When this philopatric dispersal coincides with heterogeneous habitats, an opportunity exists for the evolution of unique genetic lineages through divergent selection. The remote coral atolls in the tropical north-east Indian Ocean of northwest Australia are physically isolated from other coral reefs by hundreds of kilometres of generally slow-moving open oceanic water (Fig. 1), suggesting the strong potential for reproductive isolation and consequent local genetic diversification. Evidence from two hard corals and a coral reef fish support this expectation, indicating significant genetic differences among atolls (Underwood et al., 2009; Underwood et al., 2012; Underwood et al., 2013). Although many of the marine species of invertebrates, fish and corals present in these offshore waters have a broad Indo-West Pacific distribution, studies have demonstrated considerable divergence from congeneric populations further north and east (Horne et al., 2011; Imron et al., 2007; Otwoma and Kochzius, 2016; Underwood et al., 2018; Veilleux et al., 2010)

The coral reefs of northwest Australia are regarded as a major centre of coral biodiversity (Wilson, 2013), but little is known about the evolutionary forces that have shaped the biodiversity of most taxa on these atolls. Biological surveys suggest strong affinities between the northwest Australia atolls with the clear-water reef assemblages of Indonesia, but also highlight the diverse but under-studied nature of these remote systems (Wilson, 2013). The most comprehensive study so far recorded 1897 species (including 269 hard coral and 461 fish species), of which 262 were new records for these atolls (Bryce et al., 2009). In addition, the first survey of sponges in northwest Australia recorded 132 species, 60% of which were unique to individual reefs, and it is likely that many of these are not only endemic, but are also hitherto undescribed (Fromont and Vanderklift, 2009).

Cardinalfishes (family Apogonidae) are a major component of the fish biodiversity of coral reefs (Allen, 1993) in terms of abundance as well as species richness (Bellwood, 1996; Mabuchi et al., 2014). Cardinalfishes play an integral role in reef trophodynamics through their predominantly nocturnal, carnivorous feeding habits (Barnett et al., 2006; Bierwagen et al., 2018; Marnane and Bellwood, 2002). However, morphological similarity among species and ontogenetic changes in morphology have confused taxonomy (e.g. Fraser, 2008; Gon, 1993; Liu and Dai, 2012). Coupled with these, many apogonid species share resting sites with other species in caves and overhangs during the day (Marnane, 2000), making underwater identification of cardinalfish difficult.

Here, we investigated the genetic structure within and among populations of the Wolf Cardinalfish (*Cheilodipterus artus* Smith, 1961) from the coral atolls of northwest Australia (Fig. 1). First, to evaluate the taxonomic status and contextualise levels of divergence among our Wolf Cardinalfish samples, we sequenced the cytochrome c oxidase subunit I (CO1) gene in a subset of *C. artus* individuals alongside six other *Cheilodipterus* species. Second, to further evaluate presence of cryptic diversity among our samples from the northwest Australia atolls, we sequenced the D-loop control region of mitochondrial DNA from 58*C. artus* specimens. Third, to seek genealogical concordance and gauge the magnitude of genetic divergence between cryptic lineages identified with the mtDNA markers using nuclear genes and large sample sizes, we genotyped 593*C. artus* fish with ten microsatellite loci. Finally, we investigated the influence of habitat on genetic composition by quantifying relative wave exposure and relating this to the abundance of cryptic lineages at each sample site. Concordance across all genetic markers revealed major divergence between two cryptic lineages that live in sympatry but prefer different habitats.

## 2. Materials and methods

### 2.1. Collection and DNA extraction of northwest Australian samples

A total of 593 individuals of *Cheilodipterus artus* were collected from six sites at each of Scott Reef and Rowley Shoals in northwest Australia (Fig. 1), using a clove oil solution and hand nets by divers on snorkel or a surface supplied breathing apparatus (SSBA). Immediately after collection, fish were euthanized in an ice/seawater slurry. All fish were identified as the Wolf Cardinalfish (*Cheilodipterus artus*) using Gon (1993), and individuals were morphologically indistinguishable apart from ontogenetic changes with size that were consistent among and within sites. We also collected samples of *Cheilodipterus quinquelineatus* (Five-lined Cardinalfish) at Scott Reef and Rowley Shoals, and one of these representatives from each system was included in the phylogenetic analysis.

The habitats occupied by cardinalfish differed between Scott Reef and Rowley Shoals. At Scott Reef, Wolf Cardinalfish aggregated in the daytime under reef overhangs and caves on the slopes of the semi-enclosed deep-water lagoon (hereafter designated SDL) of the South Reef, which are sheltered from waves and currents. In addition, fish also aggregated in reef overhangs and caves on the outer reef slopes of Scott Reef (hereafter designated SOS). While providing microhabitats for shelter, these outer slope sites are more exposed to the waves and currents of the open ocean compared with the SDL sites in the deep-water lagoon. In contrast, at the Rowley Shoals, cardinalfish were only present in the fully-enclosed shallow-water lagoons (hereafter designated RSL) or at the entrance to the shallow-water lagoon in the channels (hereafter designated RSC). However, despite extensive searches by the authors, cardinalfish did not occur on the outer slopes at the Rowley Shoals. The shallow-water channel sites are not only exposed to open ocean waves, but are also flushed by oceanic water during tidal intrusions and are within a few hundred metres of the outer slope. Fish were sampled at depths of 5–30 m at Scott Reef and at depths of 5–15 m at the Rowley Shoals. Sample sizes of the entire collection ranged from 31 to 60 fish per site (Table S1).

DNA was extracted with the high throughput membrane-based DNA extraction protocol of Ivanova et al. (2006). Quality and quantity of genomic DNA was ascertained through gel electrophoresis using 1% standard agarose (Amresco, Ohio, USA). DNA was diluted by one third with millipore purified water to a final concentration of approximately 10–20 ng.

### 2.2. COI phylogenetic relationships in the Cheilodipterus genus

To contextualise genetic distances between potential lineages identified among *Cheilodipterus artus* samples collected from northwest Australia, we sequenced individuals from several *Cheilodipterus* species for the "DNA barcoding" fragment (*sensu* Hebert et al., 2003) of the mtDNA gene, cytochrome c oxidase subunit I (CO1). A subset of six *C. artus* individuals was selected to represent each of the reef systems and habitat types, as well as the full range of phylogenetic diversity apparent in each of the lineages or clades identified from the control region analysis (see below). Two *C. quinquelineatus* specimens collected from northwest Australia during this study (described above) were also included. Additional *C. artus* samples (n = −2) together with representatives of several other *Cheilodipterus* species, including *C. quinquelineatus* (n = 2), *C. macrodon* (n = 1), *C. lineatus* (n = 1), *C. isostigmus* (n = 1), and *C. novemstriatus* (n = 1), from across the Indo-West Pacific were also sequenced. These additional specimens (total n = 8) were sourced from the South African National Fish Collection (National Research Foundation – South African Institute for Aquatic Biodiversity, Grahamstown) and the University of Kansas Biodiversity Institute and Natural History Museum (GenBank accession numbers: MG950269 – MG950284; Table S2). An additional sequence of *C. intermedius* was identified from the barcoding database, BOLD Systems V.4 (http://www.barcodinglife.org; Ratansingham & Herbert 2007), and its sequence recovered from the NCBI GenBank database.

The COI fragment was amplified using the universal fish primers FishF1 and FishR1 (Ward et al., 2005) or the primer combination of Meyer (2003: dgLCO-1490 and dgHCO-2198). PCR reactions were set up in 25 uL reactions and conducted in an Applied Biosystems 2720 Thermo Cycler or an Eppendorf Mastercycler. PCR and cycling conditions were derived from Uiblein and Gouws (2014). PCR products were purified and sequenced, using standard Big Dye Terminator v3.1 (Applied Biosystems, Austin, Texas) chemistry in both directions by commercial sequencing facilities (AGRF, Perth, Australia, and Macrogen Inc., Seoul, South Korea). Sequences were aligned using ClustalX 2.0.11 (Larson and Julian, 1999), providing an alignment of 625 bp for a total of 17 specimens.

The phylogenetic relationships among species within the *Cheilodipterus* genus was inferred using Bayesian methods with Mr Bayes V3.1 (Ronquist and Huelsenbeck, 2003) and a Maximum Likelihood approach implemented within PAUP* V4.0 (Swofford, 2004). Prior to these likelihood-based analyses, jModeltest 2.1.4 (Posada and Crandall, 1998) was used to identify the most appropriate model of base and substitution frequency that best fit the sequence data using the Akaike Information Criterion (AIC; Akaike, 1974). For the Bayesian approach, four independent, simultaneous analyses were started from random trees and split frequencies of standard deviations were monitored to confirm that the Markov chains converged upon similar regions of the posterior distribution. For each analysis, four Markov chains were extended for $10^7$ generations, with every 2000th generation being sampled by the active chain. A general time reversible model with a gamma-distribution of rate variation (GTR + G) was implemented, following the outcome of the model selection procedure above, and individual model parameters estimated from the posterior distribution. The first 10% of sampled generations were discarded as "burn-in". A 75% majority-rule consensus was constructed of the post-burn in trees, with the frequencies of clades retrieved representing the posterior probabilities of them being true.

For the maximum likelihood (ML) analysis, the parameters of the model above were implemented and the optimum tree was generated using the heuristic search option (with 1000 random taxon addition replicates to generate the starting tree) implemented within PAUP*. Confidence in the resulting tree topology was assessed using 1000 bootstrap iterations (Felsenstein, 1985). Sequence divergences, corrected according to Kimura's (1980) 2-parameter model (K2P), were calculated among included representatives of each of the seven *Cheilodipterus* species, using PAUP*.

### 2.3. D-loop phylogenetic relationships within C. artus

We explored the evolutionary relationships among *Cheilodipterus artus* fish from offshore northwest Australia by a comparative analysis of the hypervariable mtDNA Control Region I (D-loop) of a subset (n = 58) of *C. artus* specimens. Using the universal primers CR-A and CR-E (Lee et al., 1995), we sequenced four to five individuals from each of the 12 sites at Scott Reef and the Rowley Shoals. PCR amplification was conducted in 25 μL reactions containing 0.2 units Ti Taq (Fisher Biotech), 2.5 μL of 10X PCR Buffer, 2.5 mM $MgCl_2$, 2.5 μL of 10 uM dNTP's, 0.4 mM of each primer and 20 ng of template DNA. PCR was performed using the following cycling parameters: 2 mins at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 48 °C and 60 s at 72 °C followed by a final extension step of 10mins at 72 °C. PCR products were purified using the Axygen (Blackburn, Australia) PCR cleanup kit and sequenced in the forward and reverse directions. These were aligned using the program Sequencher V4.8 (Gene Codes Corporation, Michigan, USA) and a consensus sequence of 387 bp was resolved for each individual.

DnaSP V5.10 (Librado and Rozas, 2009) was used to calculate haplotype and nucleotide diversity for the overall sample, as well as the average number of nucleotide differences per site (*Dxy*) between

individuals belonging to any identified clades and the fixation index ($F_{ST}$) between these clades. The phylogenetic relationships among *Cheilodipterus artus* sequences (GenBank: MG950286 – MG950343) were inferred from the D-loop alignment, using a *Cheilodipterus quinquelineatus* (MG950285) individual sequence as an outgroup. The Bayesian approach described above for the COI phylogeny was used, with the parameters of a general time reversible model with a proportion of invariable sites and a gamma distribution of rate variation (GTR + I + G) being estimated following model selection as above.

### 2.4. Nuclear genetic structure of Cheilodipterus artus

To assess whether the deep genetic divergence detected with CO1 and D-loop mtDNA sequences (see Sections 3.1 and 3.2 in Results) was discernible in the nuclear genome with faster evolving markers, we genotyped all 593 cardinalfish that were collected with 10 microsatellite loci. The genotyping procedure is described in Underwood (2010). To mitigate and report scoring error of microsatellites, quality control procedures suggested by Bonin et al. (2004) and DeWoody et al. (2006) were implemented. Specifically, we employed negative controls and visually inspected all automated allele calls, and individuals with uncertain electropherograms were repeated. A genotype error rate (1.25%) was estimated by repeating the genotyping procedure, from DNA extraction through to final allele scoring, using a subset of blind samples (n = 24) selected from three sites randomly spread across the sampling area.

To test whether divergence based on microsatellite allele frequencies were congruent with the mtDNA phylogenetic analyses, we conducted a Principal Co-ordinates Analysis (PCoA) from co-dominant, inter-individual genotypic genetic distances, implemented in GenAlEx V6.5 (Peakall and Smouse, 2006) on the subset (n = 58) of individuals that were sequenced with the D-loop mtDNA. This ordination method makes no assumptions regarding migration/drift equilibrium, mutation rate or model, and, unlike bifurcating trees, it does not have the potential to force the data into patterns that do not exist (Zink and Barrowclough, 2008).

To explore the primary level of genetic structure (optimal K) in the entire *Cheilodipterus artus* data set collected from Scott Reef and Rowley Shoals, we conducted a Bayesian clustering analysis using genotypes from all 593 fish with the program STRUCTURE V2.3 (Pritchard et al., 2000). We ran an initial analysis without prior information for $K$ = (1, 2, … 9) inferred across ten runs using the admixture model with independent allele frequencies and a burn-in of 100,000 and MCMC repetitions after burn-in of 500,000. The convergence of algorithms was checked by assessing the stability of runtime $\boldsymbol{\alpha}$ and Ln likelihood after burn-in, the variability in individual assignment proportions and the similarity score calculated with the online program CLUMPAK from ten replicate runs (Kopelman et al., 2015). CLUMPAK was also used to summarise and graphically represent the STRUCTURE results and to calculate the optimal $K$ using the $\Delta K$ method of Evanno et al. (2005) and $\ln(\Pr(X|K)$ values as per the original method of Pritchard et al. (2000). As recommended for most studies by Wang (2016), we used a separate $\boldsymbol{\alpha}$ for each population, applied an initial value of $\boldsymbol{\alpha} = 0.5$ ($1/K$ ascertained from exploratory runs) and all other parameters were left set as default values.

Once we established the individual membership for one of two lineages with microsatellite and mtDNA data sets (see Results), we conducted standard population genetics analysis on each lineage using the entire microsatellite data set. First, we assessed the variability and quality of the data by calculating the number of alleles ($N_A$), observed heterozygosity ($H_O$), the unbiased expected heterozygosity ($H_E$), and the fixation index ($F_{IS}$) at each locus and averaged across loci for each lineage with GenAlEx. Tests for linkage disequilibrium and Hardy-Weinberg Equilibrium were conducted at each site for each lineage with Genepop V4.0 (Raymond and Rousset, 1995), with default tests and Markov Chain parameters; significance levels were adjusted with sequential Bonferroni correction for multiple tests. Finally, we quantified the degree of genetic differentiation between the lineages by calculating $F_{ST}$ and $G''_{ST}$ between each lineage at each locus and across loci in GenAlEx. $G''_{ST}$ accounts for the high degree of variation within populations using microsatellite markers which often lowers the upper limit of heterozygosity values (Meirmans and Hedrick, 2011).

### 2.5. Morphology

Misclassification of *Cheilodipterus* species based on morphology is common, with uncertainty surrounding ontogenetic changes in characters (Gon, 1993). A comprehensive revision of the genus is beyond the scope of this study. However, to establish whether our specimens were indeed *Cheilodipterus artus*, and to also determine whether any major morphological differences were associated with genetic lineages 1 and 2 (see Results), we conducted basic morphological examination of representative samples using Gon (1993) and with taxonomic assistance of Gerry Allen from West Australian Museum.

### 2.6. Quantifying wave exposure

To evaluate whether the relative abundance of each lineage was influenced by habitat, we generated quantitative estimates of relative wave exposure at each sampling site using a 'Generic model for estimating relative wave exposure' (GREMO, Hill et al., 2010; Pepper and Puotinen, 2009). We extracted the typical wave exposure of sites as the frequencies and magnitude of wave energy approaching Scott Reef and Rowley Shoals from 16 equidistant compass directions from the NOAA WaveWatch III global hindcast dataset dataset (Tolman, 2009 downloaded at: http://polar.ncep.noaa.gov/waves/index2.shtml) at a spatial resolution of 0.5° and a temporal resolution of 1 h. For Scott Reef, we downloaded data for latitude 14° S and longitude 121.5° E from 7th November 2010 to 19th March 2017. For the Rowley Shoals, we downloaded data for latitude 17° S and longitude 119.5° E (Mermaid Reef), latitude 17.5° S and longitude 119.5° E (Clerke Reef), and latitude 17.5° S and longitude 119° E (Imperieuse Reef) from 7th November 2010 to 1st May 2018. Distances to the nearest wave blocking obstacle every 7.5° around each site (fetch) were weighted by the relative frequency at which waves approached the site and their average magnitude. These distances were then summed and normalised to the maximum possible summed distances (at a site 100% exposed in all directions) to create a dimensionless index of relative wave exposure. Sites inside the lagoon at Rowley Shoals were outside the model domain, and were assumed to have zero exposure. We performed a linear regression to test whether wave exposure predicted the relative abundance of *C. artus* genotypes assigned to the Lineage 1 or Lineage 2 (see Results).

## 3. Results

### 3.1. COI phylogenetic relationships in the Cheilodipterus genus

The Bayesian phylogenetic reconstruction using CO1 confirmed that the six *Cheilodipterus artus* representatives from Rowley Shoals and Scott Reef clustered within a well-supported (posterior probability = 1.00) clade together with *C. artus* specimens from the Seychelles and Zanzibar in the Western Indian Ocean (Fig. 2). This *C. artus* clade was recovered as sister to a clade containing *Cheilodipterus macrodon* and *Cheilodipterus intermedius*, with *Cheilodipterus lineatus* sister to this larger clade. This analysis also revealed three well-supported (posterior probabilities = 1.00) subclades within *C. artus*; two of these subclades comprised individuals from Scott Reef and Rowley Shoals, while a third subclade comprised the Western Indian Ocean specimens (Fig. 2). An identical pattern was obtained under ML analyses (topology not shown), implementing the parameters of the model selected above (Table S3) with ≥94% bootstrap support for the

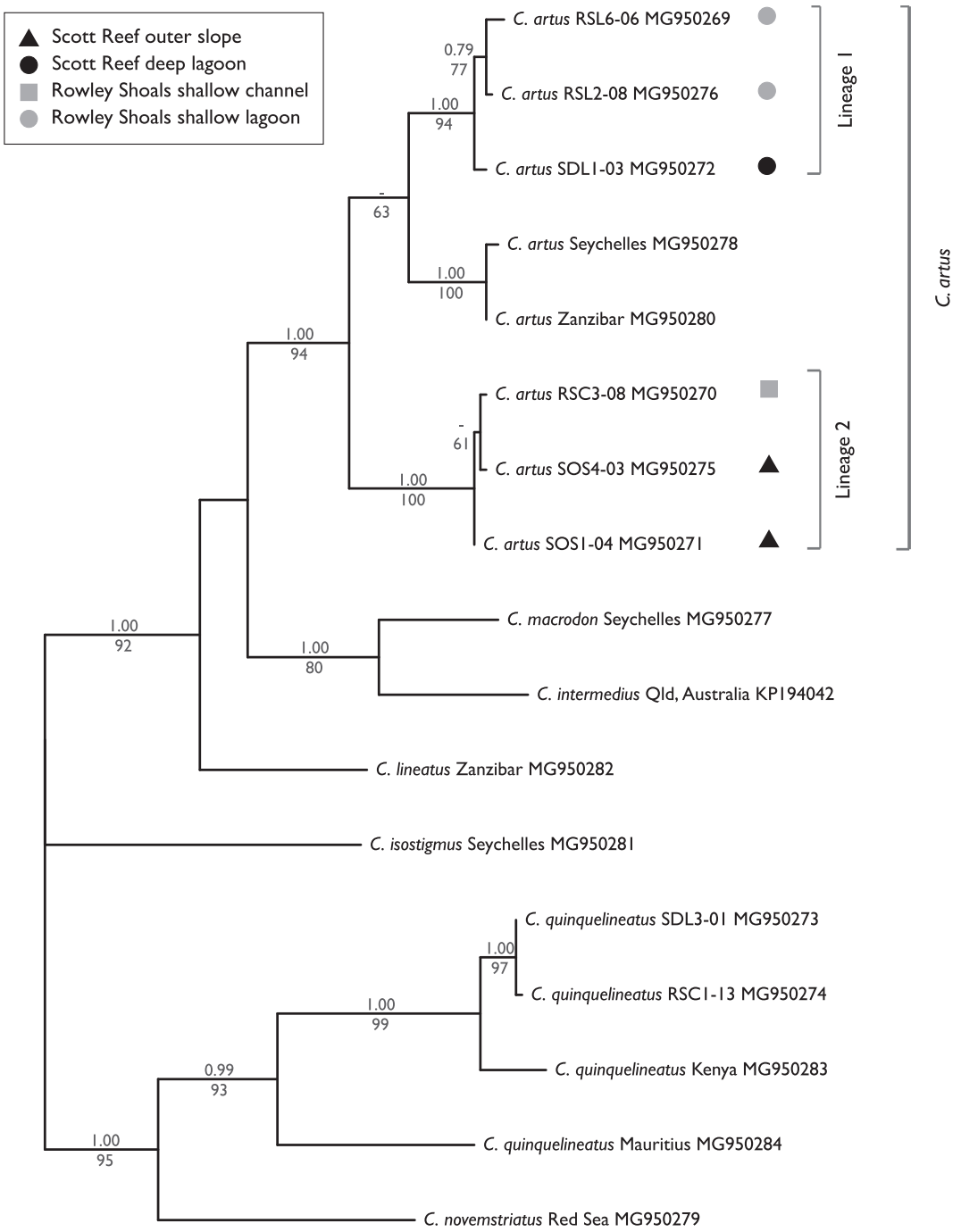


**Fig. 2.** Bayesian phylogenetic inference of *Cheilodipterus artus* representatives from sheltered and exposed habitats in northwest Australia atolls and select *Cheilodipterus* species. The topology (midpoint rooted) with the highest likelihood (lnL = −2709.511) was obtained across four MrBayes runs based on a 625 nucleotide Cytochrome c Oxidase subunit I (COI) alignment. Bayesian posterior probabilities are indicated on the branches for all clades with ≥0.75 support, with those ≥0.95 regarded as well-supported. Bootstrap values (percentages) from the maximum likelihood analysis are presented below the branches. GenBank accession numbers are given after each individual location and are presented in Table S2.

monophyly of the each of the two distinct northwest Australian clades recovered in the Bayesian analysis (Fig. 2). The first clade (from here on "Lineage 1") of the northwest Australian *C. artus* subclades comprised individuals collected from sheltered sites (RSL and SDL) on Rowley Shoals and Scott Reef, while the second clade (from here on "Lineage 2") contained individuals from exposed sites (RSC and SOS). Lineage 1 was more closely related to the WIO *C. artus* subclade compared with Lineage 2.

Representatives of additional *Cheilodipterus* species were included in these analyses to evaluate the taxonomic status of the two *Cheilodipterus artus* lineages in context to the divergence among recognised species. K2P-corrected sequence divergences between these two northwest Australian lineages ranged between 6.8% and 7.5% (mean = 7.1%; Table S4). Divergences within each northwest Australian lineage were much lower (< 1.1%). Divergence between the northwest Australian members of the Lineage 1 and the *C. artus* representatives from the western Indian Ocean ranged from 4.3 to 4.8%, while divergence between Lineage 2 and the Indian Ocean representatives was slightly higher (6.6–7.4%). Sequence divergences among the previously described *Cheilodipterus* species ranged from 7.5 to 20.7%

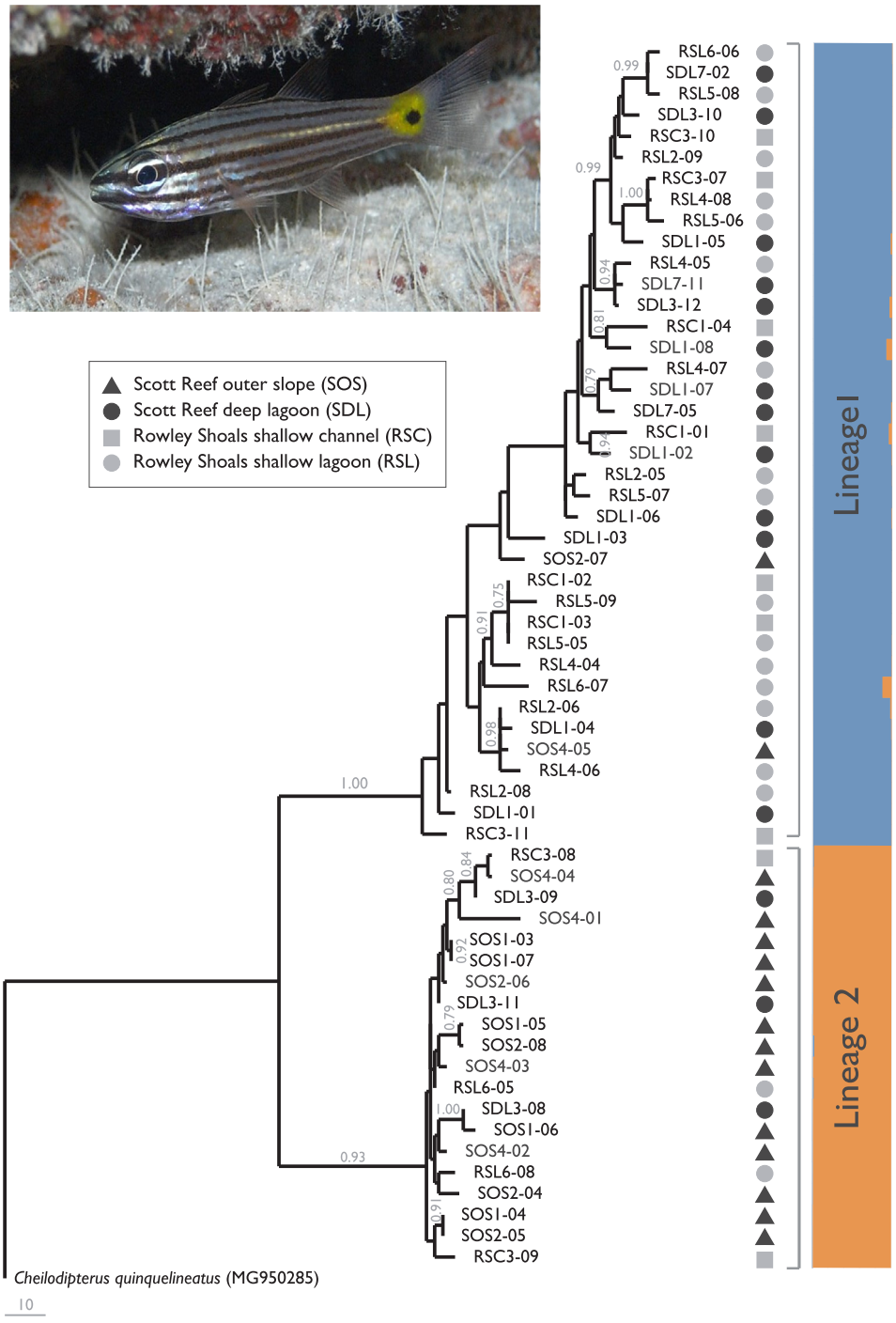


**Fig. 3.** Bayesian phylogenetic inference of relationships among 58 *Cheilodipterus artus* individuals from sheltered and exposed habitats at Scott Reef and Rowley Shoals. The topology with the highest likelihood (lnL = −2891.535) was obtained across the four MrBayes runs based on a 394 nucleotide D-loop mtDNA alignment. The tree is rooted using a sequence of *Cheilodipterus quinquelineatus* (GenBank accession number given in brackets). Bayesian posterior probabilities (≥0.75) are indicated on the branches, with values > 0.95 indicating significant support. Panel on right shows barplot from STRUCTURE clustering analysis calculated from microsatellite genotypes giving membership coefficients (*q*) of *C. artus* individuals to either the Lineage 1(blue) or Lineage 2 (orange). Inset shows picture of *C. artus* taken by authors from the shallow lagoon at the Rowley Shoals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(mean = 17.0%). K2P distances of 0. 2–11.9% were observed between *C. quinquelineatus* sequences, the only other species with multiple representatives included in the comparison.

### 3.2. D-loop phylogenetic relationships within Cheilodipterus artus

The underlying variation in D-loop mtDNA sequences in *Cheilodipterus artus* was high. The alignment of the 58*C. artus* individuals (394 bases) showed that 139 of those 387 sites not containing alignment gaps were variable; these represented 194 mutations and nucleotide diversity ($\pi$) was 0.121 ± 0.004 (SD). Fifty-four haplotypes were identified among the 58 individuals and, consequently, haplotype diversity was high (Hd = 0.997 ± 0.004). This variation provided a high power of resolution for a clear split between two *C. artus* lineages (Fig. 3). There was strong support (posterior probability = 1.00) for the monophyly of Lineage 1 (i.e. the exclusion of individuals belonging to Lineage 2 from this clade) and marginal support (posterior probability = 0.93) for the monophyly of Lineage 2. Membership to each lineage of the six representatives used in the CO1 phylogeny were the same. Relatively few individuals (< from Lineages 1 were collected from exposed habitats, and likewise, relatively few individuals from Lineage 2 were collected in sheltered habitats (Fig. 3). The average number of nucleotide substitutions per site (*D*xy) between the two lineages was 0.195, and $F_{ST}$ was 0.75. Morphological assessment of representative specimens from each lineage did not reveal any distinguishing characters and both lineages were identified as *C. artus* (pers. comm. Gerry Allen, Western Australian Museum).

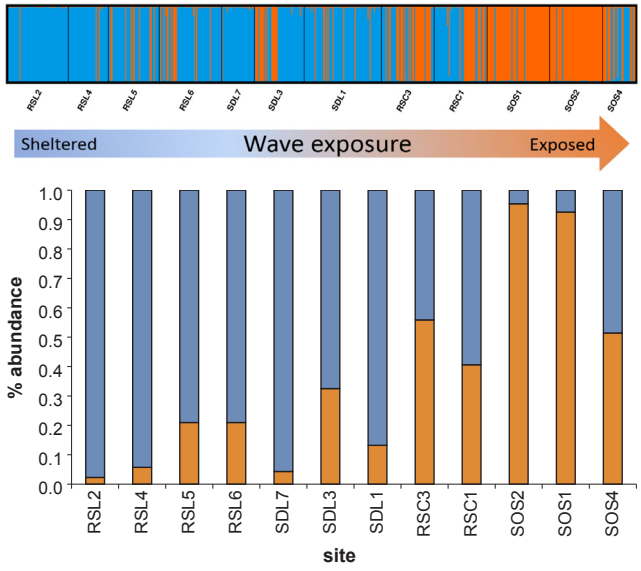


**Fig. 4.** Results of Bayesian clustering of analysis of *Cheilodipterus artus* individuals with for $K = 2$ in STRUCTURE. Sites are positioned from most sheltered to most exposed to ocean waves on x-axis. Upper panel shows that all individuals had a membership coefficient ($q$) of more than 0.75 corresponding to either cluster. Blue bar indicates the $q$ value of each individual to Lineage 1 and orange indicates $q$ value to Lineage 2. This barplot was compiled from 10/10 runs which had a similarity score of 0.999. Lower panel shows percentage abundance of the Lineage 1 (blue bars) and Lineage 2 (orange bars) at each site at Scott Reef and Rowley Shoals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.3. Nuclear genetic structure of *Cheilodipterus artus*

The microsatellite data provided unequivocal support from the nuclear genome for a split into two lineages within *Cheilodipterus artus* samples that were identified by the mtDNA sequences. The PCoA separated the subset of microsatellite genotypes which were sequenced with the D-loop marker (n = 58) into two clusters (Fig. S1), each of which corresponded to the two lineages identified in the phylogenetic analyses (Figs. 2 and 3). This split was still clearly evident when data from all 593 individuals were analysed with the Bayesian method in STRUCTURE. For $K = 2$, individuals had high membership coefficients ($q$); only 13 individuals (2%) had $q$ of less than 0.95, while all individuals had $q > 0.75$ of belonging to either cluster (Fig. 4). The division of the data into more than two clusters was not supported (Fig. S2). A total of 376 fish were assigned to the Lineage 1, and 217 fish were assigned to Lineage 2. There was 100% concordance among the multilocus microsatellite assignments and the CO1 and D-loop phylogeny; all individuals in the mtDNA data that comprised either Lineage 1 or Lineage 2 clustered together in the microsatellite STRUCTURE analysis at $q > 0.88$ (Fig. 3).

Strong differentiation was detected between the two STRUCTURE-defined lineages. Although $F_{ST}$ was moderate across all loci ($F_{ST} = 0.033$), when diversity within populations was accounted for using the method of Meirmans and Hedrick (2011), levels of differentiation were much higher ($G''_{ST} = 0.512$; Table 1). Furthermore, fixed differences were prevalent between each lineage, with private alleles observed in all loci and particularly abundant at loci B003, B104 and D137 (Fig. S3). Levels of microsatellite variation at each locus were high for both lineages, but the Lineage 1 exhibited greater diversity ($N_A = 28 \pm 3.32$ *SE* and $H_E = 0.91 \pm 0.02$ *SE*) compared with the Lineage 2 ($N_A = 22 \pm 3.09$ *SE* and $H_E = 0.85 \pm 0.04$ *SE*; Table 2). No tests for linkage disequilibrium between pairs of loci were significant after adjusting for multiple comparisons for either lineage. Hardy-Weinberg disequilibrium was detected within Lineage 1 at two loci (Ca_B107 and Ca_B125), and not at any loci within Lineage 2 (Table 2).

**Table 1**
Pairwise estimates $F_{ST}$ and $G''_{ST}$ (Meirmans and Hedrick, 2011) between Lineage 1 and Lineage 2 in *Cheilodipterus artus* for all ten microsatellite loci.

| | $F_{ST}$ | $G''_{ST}$ |
|---|---|---|
| B003 | 0.154 | 0.846 |
| D110 | 0.001 | 0.002 |
| D001 | 0.009 | 0.243 |
| B005 | 0.006 | 0.072 |
| B104 | 0.032 | 0.999 |
| D137 | 0.009 | 0.419 |
| B107 | 0.066 | 0.635 |
| B125 | 0.069 | 0.545 |
| D109 | 0.006 | 0.171 |
| D006 | 0.005 | 0.108 |
| Mean | 0.033 | 0.513 |

**Table 2**
Summary statistics of the ten microsatellites for Lineage 1 and Lineage 2 in *Cheilodipterus artus* showing the number of alleles ($N_A$), observed heterozygosity ($H_O$), expected heterozygosity ($H_E$) and fixation index ($F_{IS}$). $F_{IS}$ values in bold indicate significant departures from Hardy-Weinberg equilibrium.

| lineage | Locus | $N_A$ | $H_O$ | $H_E$ | $F_{IS}$ |
|---|---|---|---|---|---|
| 1 | B003 | 17 | 0.856 | 0.866 | 0.010 |
| | D110 | 38 | 0.957 | 0.954 | −0.005 |
| | D001 | 34 | 0.910 | 0.929 | 0.019 |
| | B005 | 22 | 0.886 | 0.881 | −0.007 |
| | B104 | 31 | 0.920 | 0.949 | 0.029 |
| | D137 | 46 | 0.957 | 0.973 | 0.015 |
| | B107 | 16 | 0.667 | 0.887 | **0.248** |
| | B125 | 14 | 0.652 | 0.737 | **0.115** |
| | D109 | 33 | 0.957 | 0.946 | −0.013 |
| | D006 | 29 | 0.949 | 0.942 | −0.009 |
| | Mean | 28 | 0.871 | 0.906 | 0.040 |
| 2 | B003 | 7 | 0.507 | 0.506 | −0.004 |
| | D110 | 36 | 0.917 | 0.951 | 0.033 |
| | D001 | 24 | 0.912 | 0.931 | 0.018 |
| | B005 | 14 | 0.802 | 0.826 | 0.026 |
| | B104 | 28 | 0.926 | 0.930 | 0.002 |
| | D137 | 31 | 0.931 | 0.949 | 0.017 |
| | B107 | 13 | 0.718 | 0.730 | 0.015 |
| | B125 | 11 | 0.783 | 0.794 | 0.011 |
| | D109 | 29 | 0.945 | 0.943 | −0.005 |
| | D006 | 24 | 0.912 | 0.919 | 0.005 |
| | Mean | 22 | 0.835 | 0.848 | 0.012 |
| Total | | 25 | 0.853 | 0.877 | 0.026 |

### 3.4. Morphology

We identified the genotyped specimens as *Cheilodipterus artus* with the following characters; large canine teeth, seven to eight dark body stripes narrower than interspaces (in adults), small dark caudal spot encircled by yellow area in smaller fish and becoming indistinct in large adults, 13 gill rakers, smooth preopercular edge and mostly 13 pectoral rays but sometimes 12 when the last ray is judged as a spine (see also inset photo in Fig. 3). In addition, there was no differences in these key characters between Lineage 1 and 2 including body stripe number, caudal spot size and appearance, preopercular edge smoothness and pectoral ray number (Fig. S4).

### 3.5. Wave exposure and lineage abundance

Exposure to wave energy varied across sites within Scott Reef and Rowley Shoals, being greatest at sites on the outer-slope of Scott Reef, intermediate at the deep-water lagoon sites at Scott Reef and the channel sites at Rowley Shoals, and least at the shallow-water lagoon

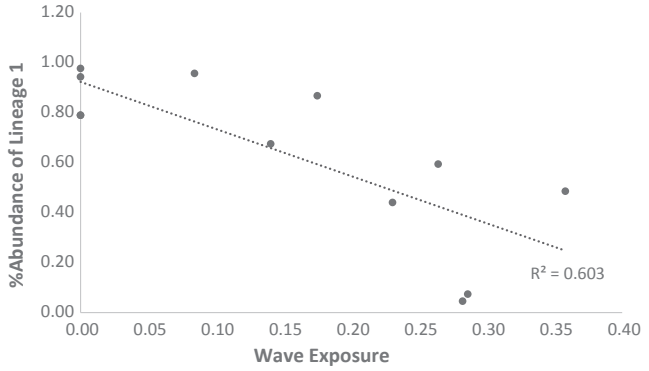


**Fig. 5.** Correlation between relative wave exposure and percentage abundance of Lineage 1 of *Cheilodipterus artus* at each site from Scott Reef and Rowley Shoals.

sites at Rowley Shoals (Fig. 1, Fig. 4 and Table S1). From 2010 to 2017, wave energy approached these systems most frequently from the SW (Figs. S5 and S6). However, the largest waves with significant wave height greater than two metres approached from the W, WNW and NW directions (Figs. S7 and S8), reflecting the influence of seasonal storms and tropical cyclones. This result explains why the outer-slope site at Scott Reef with a westerly aspect (SOS4) exhibited the greatest wave exposure.

The abundance of each *Cheilodipterus artus* lineage was associated with the degree of wave exposure at each site (Fig. 4). The most sheltered sites were dominated by individuals belonging to the Lineage 1, exposed sites were generally dominated by individuals belonging to the Lineage 2, and sites with the intermediate exposure (SDL and RSC) exhibited mixed membership. This relationship was significant ($R^2 = 0.603$; Fig. 5). The major exception was the western outer-slope site of SOS4, which was the most exposed to ocean waves but each lineage was equally abundant.

## 4. Discussion

This study identified two genetic lineages in the Wolf Cardinalfish, *Cheilodipterus artus*, from the remote coral atolls of northwest Australia that are highly divergent and morphologically cryptic. Fish from each lineage were found at the same daytime resting sites, but concordant patterns across multiple mtDNA and nuclear markers detected no evidence of hybridisation. Further, morphological examination did not detect any major phenotypic differences in key diagnostic characteristics between lineages. Despite living in sympatry, the abundance of each lineage was strongly correlated with level of wave exposure at each site, suggesting that cardinalfish from each lineage prefer different habitats to one another.

### 4.1. Cryptic divergence and reproductive isolation between lineages

The analyses of mtDNA sequences provided a broad taxonomic context to a deep genetic divergence between the two lineages in our *Cheilodipterus artus* samples of northwest Australia. The CO1 sequences revealed that all specimens identified as *C. artus* formed their own reciprocally monophyletic clade, supporting our morphological identification of these cardinalfish. However, the mean sequence divergence between the CO1 sequences of the two *Cheilodipterus artus* lineages (7.1%) not only matched those observed among some of the recognised species included in our study, but also approached (e.g. Ward et al., 2005) or exceeded (e.g. Lakra et al., 2011; Mabragaña et al., 2011) published mean inter-specific divergences for other fishes. This divergence also far exceeded typical mean intra-specific divergence values reported for the COI marker in marine fishes (Hubert et al., 2012; Mabragaña et al., 2011; Ward et al., 2005; Zhang, 2011; Zhang and Hanner, 2012). Further, fish from Lineage 1 were more closely related to *C. artus* specimens from the Western Indian Ocean than to sympatric fish from Lineage 2. This result not only indicates that genetic affinities across thousands of kilometres of open ocean are closer than those between the two lineages living in sympatry, but also that the time since divergence between the northwest Australian lineages is relatively old. The analysis of D-loop sequences that utilised larger sample sizes than CO1 analysis substantiated that the split between the two lineages was deep ($F_{ST} = 0.75$). Therefore, Lineage 1 and 2 in northwest Australia meet Mallet's (1995) and Good and Wake's (1992) operational definition of species in which genetically distinct groups live in sympatry but share stronger affinities with geographically distant populations.

Because mtDNA is maternally inherited, these CO1 and D-loop results do not preclude hybridisation at low frequencies during secondary contact but after the initial divergence. Therefore, a robust test for reproductive isolation requires nuclear genes (Avise, 2004). Here, we found no evidence of admixed ancestry with microsatellite markers. The clustering analysis unambiguously assigned all individuals to the correct lineage identified in the mtDNA trees with very high membership coefficients (Fig. 4). Moreover, fixed (diagnostic) differences were observed despite large sample sizes (n > 200 in each lineage), with private alleles abundant at several microsatellite loci. Thus, taken together, evidence from all genetic markers used indicated a long-term absence of successful hybridisation between the two lineages.

### 4.2. Ecological associations and potential mechanisms of divergence

Our study could not ascertain whether the initial divergence between the two lineages occurred in sympatry or allopatry, or whether the subsequent reproductive isolation has been maintained by ecological and behavioural (extrinsic), and/or pre- and post-zygotic (intrinsic), barriers to gene flow (Bierne et al., 2011; Knowlton, 1993; Kulmuni and Westram, 2017). Nevertheless, we hypothesise that ecological and behavioural barriers are likely to be particularly important here. Cardinalfish can accurately differentiate among their own and other conspecific local populations (Doving et al., 2006) and even mates (Rueger et al., 2018), as well as recognise natal habitats and sites (Gerlach et al., 2007; Marnane, 2000). Further, although cardinalfish vary in the degree of habitat specialisation, most are considered to be at the 'specialist' end of the scale (Gardiner and Jones, 2016). Such characteristics, along with the mouth brooding mode of reproduction, are conducive to the evolution of distinct genetic lineages through restrictions in gene flow among conspecific populations. Evidence from the east coast of Australia supports this conclusion; Gerlach et al. (2016) detected several morphologically cryptic clades within the cardinalfish *Ostorhinchus doederleini*, one of which occurred exclusively in lagoon habitat (Gerlach et al., 2016). Here, we observed a similar pattern in the Wolf Cardinalfish of northwest Australia, but there were also important differences.

The relative abundance of each lineage was significantly correlated with wave exposure. Lineage 1 was more abundant in sheltered habitats, while Lineage 2 was more abundant at those sites exposed to the open ocean waves, and each lineage was relatively evenly distributed at sites with intermediate exposure (Figs. 4 and 5). Given that coral composition is highly influenced by wave exposure (Shedrawi et al., 2017) and that cardinalfish are associated with live branching coral (Gardiner and Jones, 2016), this result suggests that the lineages may well be better adapted to particular coral reef assemblages for shelter and/or food. However, despite this association, our data shows that the niche partitioning is not complete. For example, Lineage 1 comprised 80–98% of the collection in the sheltered lagoon sites, but was always collected living side by side with at least one other fish from Lineage 2. This overlap in daytime habitat use contrasts to some degree with the exclusivity of the lagoon clade detected for *Ostorhinchus doederleini* by Gerlach et al. (2016), but is consistent with results of an apogonid study

in Papua New Guinea in which many species, including *C. artus*, exhibited a high degree of overlap in use of microhabitats (Gardiner and Jones, 2016).

Factors other than wave exposure of daytime resting sites are likely to influence selection regimes and the abundance of each lineage. Prey availability is a primary driver of resource use in cardinalfish (Barnett et al., 2006), and various cardinalfish species aggregate in the day but utilise different foraging grounds at night (Marnane and Bellwood, 2002). Further, in contrast to six other species that consumed a range of prey taxa, *C. artus* had a particularly specialised diet of only teleost fish on the Great Barrier Reef (Marnane and Bellwood, 2002). Therefore, we hypothesise that foraging habitats rather than daytime resting sites may be the primary influence on abundance of each lineage. For example, the relatively equal abundance of each lineage at the channel sites of Rowley Shoals may reflect the close proximity and accessibility of both deeper water slope habitats as well as shallow water lagoon habitats. Dietary analysis would clarify whether the night-time resource use of each lineage influences the daytime habitat association we detected here.

### 4.3. Geographic structure within lineage

In addition to the sympatric, cryptic and ecological diversity we detected, our CO1 results revealed the presence of previously unrecognised geographic structure within one of the *Cheilodipterus artus* lineages. Despite the close genetic affinities between Lineage 1 and *C. artus* from the western Indian Ocean, (relative to Lineage 2 and western Indian Ocean representatives), considerable divergence exists between these subclades, indicating the existence of geographic structure and (potentially) additional taxonomic diversity. A similar pattern was observed among the *C. quinquelineatus* specimens included in the present study, with significant geographic divergence among northwest Australia and western Indian Ocean specimens (Fig. 2). Therefore, ongoing research is required to clarify the geographic structure overlaying patterns of cryptic diversity within this taxon.

## 5. Conclusion

Cryptic diversity is prevalent in tropical reef systems (Bickford et al., 2007), and there are likely two to three times more coral reef fish species than currently recognised (Victor, 2015). Effective conservation of threatened marine ecosystems depends on accurate estimates of biodiversity, as this underpins understanding of the ecological and evolutionary processes that sustain ecosystem health and resilience (Barbosa et al., 2018; Sgro et al., 2011; von der Heyden et al., 2014). This study identified previously unrecognised evolutionarily significant units among morphologically indistinguishable *Cheilodipterus artus* cardinalfish living in sympatry on atolls in northwest Australia. Studies on other taxa in this region have revealed cryptic and ecologically important diversity in several hard corals (Gilmour et al., 2016; Rosser, 2015, 2016; Rosser et al., 2017; Underwood et al., 2018), angelfishes (DiBattista et al., 2016) and a sea snake (Lukoschek, 2018). Strikingly similar to the patterns in cardinalfish presented here, the relative abundance of distinct but cryptic lineages in the brooding coral, *Seriatopora hystrix*, also co-varied with wave exposure at Scott Reef (Underwood et al., 2018). Indeed, it appears that the evolutionary history, physical isolation and environmental heterogeneity at these atolls are particularly fertile grounds for ecological diversification. Consequently, biodiversity may be particularly underestimated in these systems: a consideration that is important for the assessment of the resilience and conservation priority of these atolls. Our results also have important ramifications for broader scientific investigations on the origins of biodiversity in the region (see Huang et al., 2018), and suggest that a taxonomic revision of the *Cheilodipterus* genus is warranted.

## 6. Research data for this article

- Names of reef systems, reefs and sites, GPS coordinates of sites, date of collection and sample sizes of *Cheilodipterus artus* collections used for the microsatellite genotyping are given in Table S1.
- All sequences used in CO1 genus phylogeny have been lodged in GenBank and accession numbers given in Table S2.
- Microsatellite genotypes lodged in DRYAD (doi:10.5061/dryad.47sd969) and new D-loop sequences lodged in Genbank (submission numbers MG950269 to MG950343).

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2018.12.001.

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